IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

RE PATENT OF :

TOSHIKAZU YOSHIKAWA ET AL : GROUP ART UNIT: 1623

SERIAL NO: 09/890,562 : EXAMINER: Kathleen Kahler Fonda

FILED: AUGUST 1, 2001 :

FOR: AGENT FOR PREVENTING AND CURING ARTERIOSCLEROSIS

DECLARATION UNDER 37 C.F.R. §1.132

HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231

Being duly sworn, I, Norimasa Yoshida, a citizen of Japan, residing at 76, Shimogamokitazono-cho, Sakyou-ku, Kyoto-shi, Kyoto-fu, Japan depose and say:

I. I am one of co-inventors in the above referenced application, and a doctor of medicine as well as belonging to Molecular Gastroenterology and Hepatology, Graduate School of Medical Science of Kyoto Prefectural University of Medicine.

I graduated from Kyoto Prefectural University of Medicine, School of Medicine, in March 1980.

From 1985 to 1990, and from 1995 to March 2003, I was the employ of First Department of Internal Medicine of this University, from April 2003 up till the present, I have been

the employ of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science of this University, and I have been engaged in the research work of free radical, cytokine and cell adhesion molecule in inflammation and immune disorder, and I am now an assistant professor.

I am well acquainted with all the co-inventors in this case, having worked with them on the development thereof, and in this application I am expressing their opinion, as well as my own.

II. In order to investigate the effects of the chromanol glucoside (TMG) on the expression of the cell adhesion molecule (VCAM-1 and ICAM-1) which is the important factor of the development of arteriosclerosis, I made the experiments as follows:

EXPERIMENT

<Culture of human aortic endothelial cells>

Human aortic endothelial cells (HAEC) were purchased by CAMBREX (MD, USA). HAEC were plated in Medium 199 (GIBCO, Great Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories Inc., Logan, Utah), thymidine (2.4mg/mL; Sigma Chemical, St. Louis, MO), glutamine (230mg/mL, JRH Bioscience), heparin sodium (10IU/mL, Sigma), antibiotics (100 IU/mL peniciline, 100mg/mL streptomycin, and 0.125μg amphotericin B), and endothelial cell growth factor (80mg/mL, Biomedical Techologies Inc., Stoughten, MA). The cell cultures were incubated at 37C in a humidified atmosphere with 5% CO2 and expanded by brief

trypsinization (0.25% trypsin in phosphate-buffered saline (PBS) containing 0.02% ethylenediamine tetraacetic acid). HAEC from the primary through the second passage were prepared on 96-well tissue culture plates that were coated with gelatin (0.1%) and fibronectin-coated (25mg/mL), and used when confluent.

< Monoclonal antibodies>

The blocking monoclonal antibodies (MAbs) directed angaist ICAM-1 (LB-2) and VCAM-1 (E1/6) were supplied by Becton Dickinson (San Jose, CA).

<Enzyme immunoassay (EIA)>

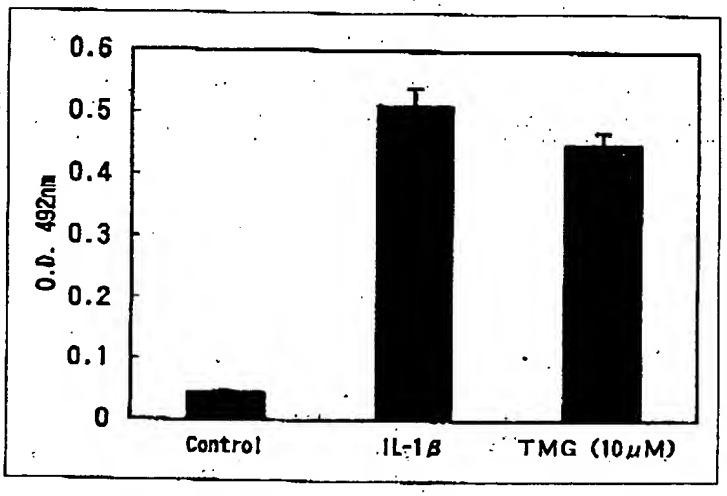
EIA was used to assess the binding of MAbs to the endothelial cell monolayers. Confluent HAEC monolayers prepared in 96-well plates were pretreated with Medium 199 supplemented with TMG (final concentration of 10mM). 12h later, HAEC were washed twice with HBSS to remove TMG, and then were stimulated by IL-1β (20U/ml). 6h later, HAEC were fixed by addition of 1% paraformaldehyde in PBS for 15 min at room temperature. The wells were washed three times with phosphate-buffered saline (PBS) and incubated in 2% bovine serum albumin (BSA) for 30 min. After removing BSA, MAb directed against ICAM-1 or VCAM-1 was added and incubated at 37C for 1h, washed three times in PBS, and then incubated for 1h in peroxidase-conjugated goat-anti-mouse IgG affinity-purified F(ab')2 fragment (Cappel, Durham, NC). After washing, substrate (o-phenylenediamine dihydrochloride, 0.4mg/mL, in buffer, pH 5.0) was added and incubated for 30 min at room temperature. The plates were then read at 492nm in a Micro plate reader (Tosoh, ToKyo) to quantitate the

amount of bound antibody.

The results of these tests are shown in the following Tables and Diagrams.

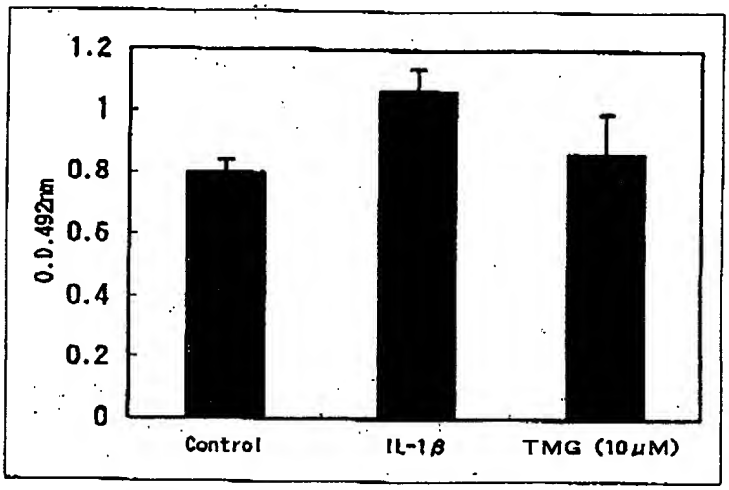
<Effect of TMG on increased expression of VCAM-1 on HAEC induced by IL-1β>

	Average(n=3)	Standard Error
Control	0.043	0.004
IL-1β	0.512	0.028
TMG(10μM)	0.449	0.019



<Effect of TMG on increased expression of ICAM-1 on HAEC induced by IL-1β

	Average(n=3)	Standard Error
Control	0.043	0.004
IL-1β	0.512	0.028
TMG(10μM)	0.449	0.019



III. CONCLUSION

As being clear from the results of EXPERIMENT, TMG evidently suppressed the increased expression of VCAM-1 and ICAM-1 on HAEC induced by IL-1β. Consequently, it has been proven that expression inhibitory effect of cell adhesion molecule is greatly concerned with the action mechanism of the arteriosclerotic treatment of chromanol glucoside.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statement and the lie so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

DATE: Dec. 29, 2003

N. Yoshida

Norimasa Yoshida